BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

REMARKS

This is a full and timely response to the Office Action mailed March 24, 2004.

By this Amendment, claims 8-12, 14-15 and 17-19 were cancelled without prejudice or disclaimer to their underlying subject matter, and new claims 20-32 were added. The new claims correspond to the same subject matter presented in the canceled claims. Thus, support for the new claims can be found variously throughout the specification and original claims. Claims 20-32 are pending in this application.

In view of this Amendment, Applicant believes that all pending claims are in condition for allowance. Reexamination and reconsideration in light of the above amendments and the following remarks is respectfully requested.

Rejections under 35 U.S.C. §102

Claims 8-11 are rejected under 35 U.S.C. §102(b) as allegedly being anticipated by Sha et al (Immunobiology, p. 21 - 30 (1999). Applicant respectfully traverses this rejection.

To constitute anticipation of the claimed invention, a single prior art reference must teach each and every limitation of the claims. Here, in this case, Sha et al. fails to teach a liposomal vaccine composition comprising a plasmid **encapsulated within a liposome**.

The Examiner has taken the position that Sha et al. teaches a DNA vaccine using an influenza HA gene that is "*encapsulated in liposomes*". The Examiner believes that since the lipid formulation in Sha et al. are mixed with DNA in the same manner as in the present invention, the DNA of Sha et al. must be encapsulated within the liposome in the same manner as in the present invention. Further, the Examiner believes that there is no distinction between DNA being "*complexed*" with liposome and DNA being "*encapsulated*" within liposomes. However, Applicant strongly disagrees with the Examiner in this regard since the Examiner's position contradicts what is known in the art.

In Xu et al. (Physicochemical Characterization and Purification of Cationic Lipoplexes, Biophysical Journal, vol. 77, July 1999, pages 341-353) (enclosed herewith), the authors explain on page 341, right column, that:

> "*Cationic liposomes bind to DNA and form clusters of aggregated vesicles on the DNA (i.e. complexes of DNA with lipids). When the charge ratio (+/-)*

> *approaches 1:1, the membranes of the clustered liposomes go on to fuse. This results in mixing of lipids from adjacent liposomes and simultaneous collapse of the DNA to a condense structure. The process is highly cooperative and was suggested to lead to* ***DNA encapsulation inside an enclosed bilayer membrane***."

Such a teaching can also be found in other references in the art. For example, in Baubak Bajoghli (The Use of Synthetic Polymers for Delivery of DNA, enclosed herewith), the author states:

> *"Cationic liposomes are able to interact spontaneously with negatively charged DNA to form clusters of aggregated vesicles along the nucleic acid (i.e. complexes of DNA with lipids). At a critical liposome density, the DNA is condensed and* ***becomes encapsulated within a lipid bilayer***."

Thus, given these clear teachings in the art, there is a distinction between DNA being "*complexed*" (or clustered) with liposome and DNA being "*encapsulated*" within liposomes.

Further, the Examiner's statement that the lipid formulation in Sha et al. are mixed with DNA in the same manner as in the present invention is also incorrect since it fails to compare the ratio at which the DNA and lipid are mixed.

In Sha et al., the influenza HA gene is mixed with lipid at a ratio of 1 to 2 (see page 22 of Sha et al., "*20μg of pjw4303 DNA was mixed with 40μg of Dosper in HBS*"), and thus, is complexed with liposomes and not encapsulated. On page 26 of Sha et al., the authors discuss that cationic liposomes have been shown to efficiently complex with DNA. In other words, the experiments of Sha et al. were conducted to determine the effectiveness of DNA/lipid complexes and not composition of DNA encapsulated within liposomes.

In contrast, the present specification teaches that the plasmid DNA is added to the lipid film at a concentration of 400μg DNA/ml of 10mg/ml (see page 10, lines 15-16, of the specification). This translates to a DNA to lipid ratio of 1 to 25 which is clearly different from the ratio taught in Sha et al.

This difference in ratio between the present invention and Sha et al. results in *the DNA being condensed and* ***becoming encapsulated within a lipid bilayer membrane.***" Such a structure is not taught or suggested in Sha et al.

Further, based on the teachings and experiments of the specification (as discussed in the Declaration), it is clear that the difference in liposomal formulation between Sha et al. and

the present invention is the key reason why the presently claimed composition can elicit long-lasting protective antiviral immune responses against influenza viruses as compare to the composition of Sha et al.

It is stated in Sha et al. that their experiment did not protect the mice against the challenge, and that their method of challenge (intramuscular injection) bypassed the mucosal antibodies that were induced by their (liposomal) vaccine. In contrast to Sha et al., intramuscular injection of the presently claimed liposomal vaccine composition provided complete 100% protection in mice (see Fig. 4) (as compared to 0% protection in Sha et al.), but yet did not induce mucosal antibodies. This clearly demonstrates that the liposomal vaccine composition of the present invention (i.e. *plasmid encapsulated within a liposome*) is fundamentally distinct in terms of structural makeup, biological activity and function from the DNA/liposome complex of Sha et al.

Thus, for these reasons, withdrawal of this rejection is respectfully requested.

It should also be noted that given the poor results shown in Sha et al. and the clearly unexpected and superior results of the present invention, a case of obviousness also cannot be made.

Rejections under 35 U.S.C. §103

Claims 8, 12, 14, 15 and 17-19 are rejected under 35 U.S.C. §103(a) as being unpatentable over Sha et al. and Promega Catalog (Technical Bulletin 206, rev. 7/1999).

To establish a *prima facie* case of obviousness, the cited references, in combination, must teach or suggest the invention as a whole, including all the limitations of the claims. Since for the reasons noted above, the combination of Sha et al. and Promega Catalog fails to teach or suggest a liposomal vaccine composition comprising a plasmid **encapsulated within a liposome,** this rejection also cannot be sustained and should be withdrawn.

CONCLUSION

For the foregoing reasons, all the claims now pending in the present application are believed to be clearly patentable over the outstanding rejections. Accordingly, favorable reconsideration of the claims in light of the above remarks is courteously solicited. If the Examiner has any comments or suggestions that could place this application in even better form, the Examiner is requested to telephone the undersigned attorney at the below-listed number.

Respectfully submitted,

Dated: June 22, 2004

By 
Lee Cheng
Registration No.: 40,949

RADER, FISHMAN & GRAUER PLLC
The Lion Building
1233 20th Street, N.W., Suite 501
Washington, DC 20036
(202) 955-3750
Attorney for Applicant

Should additional fees be necessary in connection with the filing of this paper, or if a petition for extension of time is required for timely acceptance of same, the Commissioner is hereby authorized to charge Deposit Account No. 180013 for any such fees; and applicant(s) hereby petition for any needed extension of time.

Biophysical Journal Volume 77 July 1999 341–353

Physicochemical Characterization and Purification of Cationic Lipoplexes

Yuhong Xu,*# Sek-Wen Hui,# Peter Frederik,§ and Francis C. Szoka, Jr.*

*University of California, School of Pharmacy, Department of Biopharmaceutical Sciences and Pharmaceutical Chemistry, San Francisco, California 94143-0446 USA; #Roswell Park Cancer Institute, Membrane Biophysics Lab, Buffalo, New York 14263 USA; and §Department of Pathology, University of Limburg, Maastricht, the Netherlands

ABSTRACT Cationic lipid-nucleic acid complexes (lipoplexes) consisting of dioleoyltrimethylammoniumpropane (DOTAP) liposomes and plasmid DNA were prepared at various charge ratios (cationic group to nucleotide phosphate), and the excess component was separated from the lipoplex. We measured the stoichiometry of the lipoplex, noted its colloidal properties, and observed its morphology and structure by electron microscopy. The colloidal properties of the lipoplexes were principally determined by the cationic lipid/DNA charge ratio and were independent of the lipid composition. In lipoplexes, the lipid membranes as observed in freeze-fracture electron microscopy were deformed into high-radius-of-curvature features whose characteristics depended on the lipid composition. Lipoplexes prepared at a threefold or greater excess of either DOTAP or DNA could be resolved into complexes with a defined stoichiometry and the excess component by sedimentation to equilibrium on sucrose gradients. The separated, positively charged complex retained high transfection activity and had reduced toxicity. The negatively charged lipoplex showed increased transfection activity compared to the starting mixture. In cryoelectron micrographs the positively charged complex was spherical and contained a condensed but indistinct interior structure. In contrast, the separated negatively charged lipoplexes had a prominent internal 5.9 ± 0.1-nm periodic feature with material projecting as spikes from the spherical structure into the solution. It is likely that these two lipoplexes represent structures with different lipid and DNA packing.

INTRODUCTION

Cationic lipids have become a major research tool for transferring DNA into cells in vitro and are being investigated in a variety of gene therapy trials in humans (Felgner et al., 1987; Gao and Huang, 1995). In this simple protocol, cationic liposomes are mixed with DNA to form a cationic lipid-DNA complex (designated *lipoplex*; Felgner et al., 1997). For in vitro applications, lipoplexes are usually formed at excess positive charge ratios (cationic moiety to nucleotide), and the resulting complexes are applied to cells (Felgner et al., 1987, 1994; Gao and Huang, 1991). For in vivo applications, lipoplexes formed at excess positive and excess negative charge ratios have been used for gene transfer (Gao and Huang, 1995). Despite this widespread use in biology, the most critical factor determining lipoplex transfection activity is not clear.

Lipoplexes can be defined with two major parameters: the lipid composition and the charge ratio between the lipid and DNA. To identify the principal determinant of high transfection, a clear understanding of the correlation among these parameters and the resulting colloidal properties and of the structure of the lipoplexes is required.

Gershon and co-workers were the first to study the interaction between DOTMA/DOPE liposomes and DNA and the structures arising when they were mixed (Gershon et al., 1993). They suggested that cationic liposomes bind to DNA and form clusters of aggregated vesicles on the DNA. When the charge ratio (±) approaches 1:1, the membranes of the clustered liposomes go on to fuse. This results in mixing of lipids from adjacent liposomes and simultaneous collapse of the DNA to a condensed structure. The process is highly cooperative and was suggested to lead to DNA encapsulation inside an enclosed bilayer membrane. Electron micrographs of metal-shadowed cationic lipid/DNA complexes revealed rod-shaped structures interpreted to be inside liposomes at a charge ratio of 1:1. The membrane fusion hypothesis was consistent with studies reporting fusion between cationic liposomes mediated by various polyanions (Duzgunes et al., 1989; Keren-Zur et al., 1989) or with anionic liposome and cells (Stamatatos et al., 1988; Leventis and Silvius, 1990). These and other studies reinforce the hypothesis that the inclusion of fusogenic lipids such as DOPE in the lipoplex is critical to transfection activity (Felgner et al., 1994; Farhood et al., 1995).

Sternberg and co-workers were the first to publish freeze-fracture electron micrographs depicting the lipid morphology arising when cationic liposomes composed of DC-cholesterol and DOPE (1:1 molar ratio) interacted with DNA (Sternberg et al., 1994). Aggregated and semifused liposome structures were observed at a low DNA-to-lipid ratio or after a short incubation time. At cationic lipid/DNA charge ratios close to 1:1, they observed complexes containing tubular structures with a 7-nm diameter. They proposed a model for the structures that they termed the "spaghetti (DNA helix inside a lipid tube) and meatball (aggregated liposomes) model." The lipid tubules were observed at charge ratios quite close to that used for optimal transfection, leading them to conclude that the tubule structures were the active lipid-DNA assembly structure.

Received for publication 4 August 1997 and in final form 12 April 1999.

Address reprint requests to Dr. Francis C. Szoka, Jr., School of Pharmacy, University of California–San Francisco, Department of Biopharmaceutical Sciences and Pharmaceutical Chemistry, San Francisco, CA 94143-0446. Tel.: 415-476-3895; Fax: 415-476-0688; E-mail: Szoka@cgl.ucsf.edu.

0006-3495/99/07/341/13 $2.00

Cryotransmission electron micrographs of complexes formed from various cationic detergents and lipids with DOPE were examined by Gustafsson and co-workers (1995). They observed that discrete clusters of aggregated particles appeared with the addition of DNA to cationic liposomes with a charge excess of cationic lipid to DNA. The various lipids and detergents examined formed complexes with DNA that consisted of a compact striated structure that was interpreted as "a compact and disordered multilamellar structure." There was no evidence of discrete tubular structures in the cryoelectron micrographs. With an excess of DNA, there appeared to be strands of DNA protruding from the complex.

More recently, two groups have applied small-angle x-ray diffraction (SAXS) to the investigation of the structures of the complexes (Lasic et al., 1997; Radler et al., 1997). Lasic and co-workers used a combination of cryoelectron microscopy and SAXS to examine a complex formed from dioctadecyldimethylammonium/cholesterol (1:1 molar ratio) and plasmid at a 2:1 (±) charge ratio. They concluded that the DNA was absorbed between lamellar sheets as a single layer of parallel helices. The spacing determined by both methods was 6.5 nm, which is consistent with the dimensions of a bilayer and a hydrated DNA helix. Radler and colleagues examined the SAXS of lipoplexes formed between liposomes composed of DOTAP/DOPC at various molar ratios and plasmid DNA. The SAXS pattern showed a clear periodic structure with a 6.5-nm spacing, which was fairly constant regardless of the lipid/DNA charge ratio used to prepare the lipoplex. There was another peak, which they interpreted as the DNA interaxial spacing. This spacing changed with the lipid/DNA charge ratio and with the cationic lipid/neutral lipid ratio. At charge ratios higher than 1:1 it was 5.7 nm, and at charge ratios lower than 1:1 it was 2.4 nm. From these values they postulated a lipoplex structure consisting of lipid multilayers with DNA between the layers.

Based upon the above studies, it appears that lipoplexes formed at an excess negative charge ratio differ from those formed at an excess positive charge ratio in their colloidal properties as well as in their lipid/DNA packing structures. However, this aspect is not yet resolved, because most of the studies were based on one cationic liposome composition prepared at a neutral or positive charge ratio. We were interested in learning whether different liposome compositions would result in complexes with different colloidal properties or different lipoplex structures. We show that the lipid composition in the liposomes is irrelevant to the colloidal properties of the complex, but composition strongly influences the microscopic structure of the complexes. High-radius-of-curvature structures were observed in transfection active lipoplexes.

We found two distinctively different lipid/DNA packing structures: one found at excess positive charge ratio, the other found at excess negative charge ratio. We were curious to learn whether they represent a continuum of structures that have unique characteristics at each charge ratio or, rather, if there are two stoichiometric assemblies formed under conditions of an excess component. We demonstrated that lipoplexes with a defined stoichiometry can be prepared when one of the reactants is provided in more than a threefold excess and separated from unreacted excess component by sedimentation to equilibrium on sucrose gradients. These isolated, stoichiometric complexes had a small diameter and were homogenous and relatively stable to storage. Furthermore, both the negative and positive complexes were active in transfection.

MATERIALS AND METHODS

Materials

Dioleoyltrimethylammoniumpropane (DOTAP), dioleoylphosphatidylethanolamine (DOPE), and dioleoylphosphatidylcholine (DOPC) were purchased from Avanti Polar Lipids (Alabaster, AL). The fluorescence-labeled lipids rhodamine-phosphatidylethanolamine (Rh-PE) and *N*-(7-nitrobenz-2-oxz-1,3-diazol-4-yl) dipalmitoylphosphatidylethanolamine (NBD-PE), as well as the DNA intercalating dye ethidium bromide (EtBr), were from Molecular Probes (Eugene, OR). Plasmid DNA (7.6 kb) encoding β-galactosidase as the reporter gene was prepared by a standard protocol (Sambrook et al., 1989) and routinely had 260 nm/280 nm absorbance ratios greater than 1.8 and was predominantly supercoiled. DNA concentration was determined using a value of 50 μg DNA per one absorbance unit at 260 nm. DNAse I was obtained from Gibco BRL (Gaithersburg, MD). $C_{12}E_8$ was from CalBiochem (San Diego, CA). All of the other reagents, including monooleoylglycerol (MOG), cholesterol (Chol), and *O*-nitrophenyl-β-D-galactopyranoside (ONPG) were obtained from Sigma (St. Louis, MO).

Sample preparation

To prepare liposomes, a chloroform solution of lipids was placed in a glass vessel, and the chloroform was removed under vacuum while the lipid was deposited on the side of the vessel. The lipid film was exposed to a high vacuum for at least 2 h. The lipids were resuspended in water, and the suspension was irradiated in a bath-type sonicator (Lab Supplies, Hicksville, NY) under argon until a translucent lipid suspension (<30 min) was obtained (Legendre and Szoka, 1992). Liposomes or, in some cases, micelles formed by this method had a diameter between 10 and 80 nm. The exact value is indicated in the figure legends. Cationic liposome/DNA complexes were made by mixing equal volumes of the liposome and DNA at the lipid positive charge/nucleotide negative charge ratio indicated. For complexes prepared with an excess positive charge ratio or at the charge equivalence point, the DNA was rapidly injected into the cationic liposome suspension. For complexes prepared with an excess negative charge ratio, the cationic liposome suspension was rapidly injected into the DNA solution. The salt concentration in the buffer was always equal to or less than 30 mM Tris/HCl, and the final DNA concentration varied between 12 and 500 μg/ml. The exact DNA concentration is indicated where appropriate.

Size and zeta potential measurement

The particle size of the liposomes and their complexes with DNA were obtained by dynamic light scattering measurements on a sub-Micron Particle Analyzer (Coulter N4; Coulter Electronic). The zeta potentials were determined with a laser electrophoretic mobility instrument Zetasizer (model 4; Malvern Instruments) in a 30 mM NaCl buffer. The zeta potential and diameter of the lipoplexes were measured within 3 h of their formation.

Lipid mixing measurements

To measure the rate and extent of lipid mixing during complex formation, we used the assay of Struck and Pagano (Stuck et al., 1981). Cationic liposomes containing 1 mol% of Rh-PE and NBD-PE were mixed at a 1:8 ratio with unlabeled cationic liposomes. Because of the fluorescence energy transfer, NBD emission (I_0) was greatly reduced in close proximity to Rh-PE. With the addition of DNA to the cationic liposomes, lipid mixing between labeled and unlabeled liposomes resulted in the dilution of the fluorescence label and an increase in NBD-PE fluorescence. The increase in NBD emission (I) was used to calculate the percentage of lipid mixing:

$$\% \text{ of lipid mixing} = \frac{I - I_0}{I_{max} - I_0} \times 100\ \% \qquad (1)$$

where I_{max} is the maximum dequenching of the NBD emission obtained by dissolving liposomes with the detergent $C_{12}E_8$. The fluorescence intensity is recorded using a Spex Fluorimeter (excitation wavelength: 470 nm; emission wavelength: 527 nm; 2.5-mm excitation and 5.0-mm emission slits). The reproducibility of the lipid mixing measurement was ±5%.

Displacement of EtBr intercalation

EtBr fluorescence at 610 nm was recorded in a Spex Fluorimeter (excitation wavelength: 500 nm) to measure the effect of cationic liposome binding on EtBr intercalation into DNA. Fluorescence intensity obtained from a plasmid DNA solution (5 μg/ml) containing 0.2 μg/ml EtBr is defined as the maximum intensity (100%). After the mixing of cationic liposomes with DNA, the resulting fluorescence intensity was compared to the maximum intensity, and the percentage change was used as an indication of the EtBr displacement. The values reported are steady state values obtained in less than 3 min after the liposomes are mixed with the DNA. The data are not corrected for any light scattering due to addition of liposomes. Light scattering caused less than a 5% change in the fluorescence signal.

Susceptibility of DNA to DNase I digestion

To determine whether DNA is exposed to the aqueous environment when complexed with cationic liposomes, we measured the susceptibility of DNA to degradation by DNase I. The complexes were incubated with DNase I (2 units/μg of DNA) in reaction buffer (0.9 mM $MnCl_2$, 50 mM Tris-HCl, pH 7.4) for 5, 30, and 90 min. At each time point, the reaction was stopped by adding an equal volume of phenol. The sample was then extracted two times with chloroform/high-salt buffer to remove the bound lipids. The aqueous solution containing the DNA and/or digestion products was loaded directly onto a 1% agarose gel and separated in an electric field of 110 v for 2 h to examine the integrity of the plasmid DNA.

Purification of cationic liposome/DNA lipoplexes

To separate cationic liposome/DNA complexes from unbound or loosely bound liposomes or DNA, lipoplexes mixed at different charge ratios were loaded on top of a 4-ml 0–30% (w/w) linear sucrose gradient. After 16 h of ultracentifugation (40,000 rpm in a Beckman rotor SW 50.1; 150,000 × g), the complexes migrate to a specific density that was either visible to the eye or could be detected by chemical analysis. To analyze the distribution of the complexes, the gradient was separated into 10 fractions, and each fraction was assayed for lipid and DNA content. To determine the lipid concentration, 1% fluorescent label Rh-PE was added to the liposomes, and the fluorescence after $C_{12}E_8$ solubilization of the liposomes was used to quantify lipid concentration. The plasmid DNA concentration in each fraction was measured either using the Hoescht dye 33258 (Polysciences, Warrington, PA) and a DNA fluorimeter (TKO100; Hoeffer Scientific Instruments, San Francisco, CA) (sensitivity >10 ng/ml DNA) after methanol/chloroform extraction of the mixture, or by using radiolabeled plasmid DNA.

Freeze-fracture electron microscopy of lipoplex suspensions

The morphology of the liposomes and lipoplexes was examined by electron microscopy after freeze-fracture sample preparation as previously described (Hui, 1988). About 2 μl of the cationic liposome/DNA lipoplex sample (at 200 μg/ml or higher DNA concentration) was sandwiched between two copper plates, and the sample was rapidly frozen by plunging it quickly into liquid propane. The sample was then fractured and shadowed with carbon (0° tilt) and platinum (45° tilt) at −110°C and 2×10^{-6} torr high vacuum. The replicas were cleaned and examined in a transmission electron microscope (Hui, 1988).

Cryoelectron microscopy of lipoplex suspensions

The samples for cryoelectron microscopy were prepared at 100 μg/ml DNA. A thin aqueous film was formed on a grid with a fine honeycomb pattern (700 mesh) that was 3–4 μm thick. The thin film was rapidly vitrified by plunging the grid into ethane cooled to its melting point by liquid nitrogen. The vitrified sample grid was stored under liquid nitrogen during transfer to the cold stage of the electron microscope (Philips CM30). The micrographs were taken with an acceleration voltage of 100 kV at −170°C at various defocus values between −0.9 and − 2.1 μm as previously described (Frederik et al., 1991).

Cell transfection

Cationic liposome/DNA lipoplexes with different lipid formulations and charge ratios were examined for in vitro transfection activity in CV1 cells (monkey fibroblast-like cells). The β-galactosidase gene is used as the reporter gene (pCMV-β-gal) and was a generous gift of Dr. McGregor (McGregor and Caskey, 1989). Cells were plated in a 96-well cell culture dish 24 h before transfection at 20,000 cells/well and grown in DME-H21 media containing 10% fetal calf serum at 37°C, 5% CO_2. Immediately before transfection, the cell monolayer was rinsed once with serum-free medium and medium containing 0%, 10%, 20%, or 30% serum placed over the cells. Preformed complexes were added to the cells (0.6 μg DNA/well unless otherwise indicated) in a volume of 50 μl and incubated for 4 h. The incubation medium was then replaced by medium containing 10% fetal calf serum, and cells were incubated for another 40 h. The transfection activity was measured as previously described (Tang et al., 1996) and reported as the amount of β-galactosidase protein activity obtained per well, using enzyme-linked immunosorbent assay-grade β-galactosidase from CalBiochem (San Diego, CA) as the standard.

RESULTS

Characterization of cationic lipoplexes

Size and zeta potential of lipoplexes

Cationic liposomes with different lipid compositions were mixed with plasmid DNA at various charge ratios, and the diameter and zeta potential of the resulting complexes were measured (Fig. 1). The absolute value of the mean diameter of the lipoplexes was a function of the lipid and DNA concentration during mixing, the ionic strength of the solution, the mixing rate, and the amount of mechanical agitation applied to the suspension (data not shown). Thus a standard protocol that involved injecting equal volumes of the minor component and the component in excess in a polystyrene tube was used to prepare all of the combinations. The salt concentration in the buffer was always equal to or less than 30 mM Tris/HCl, and the final DNA con-

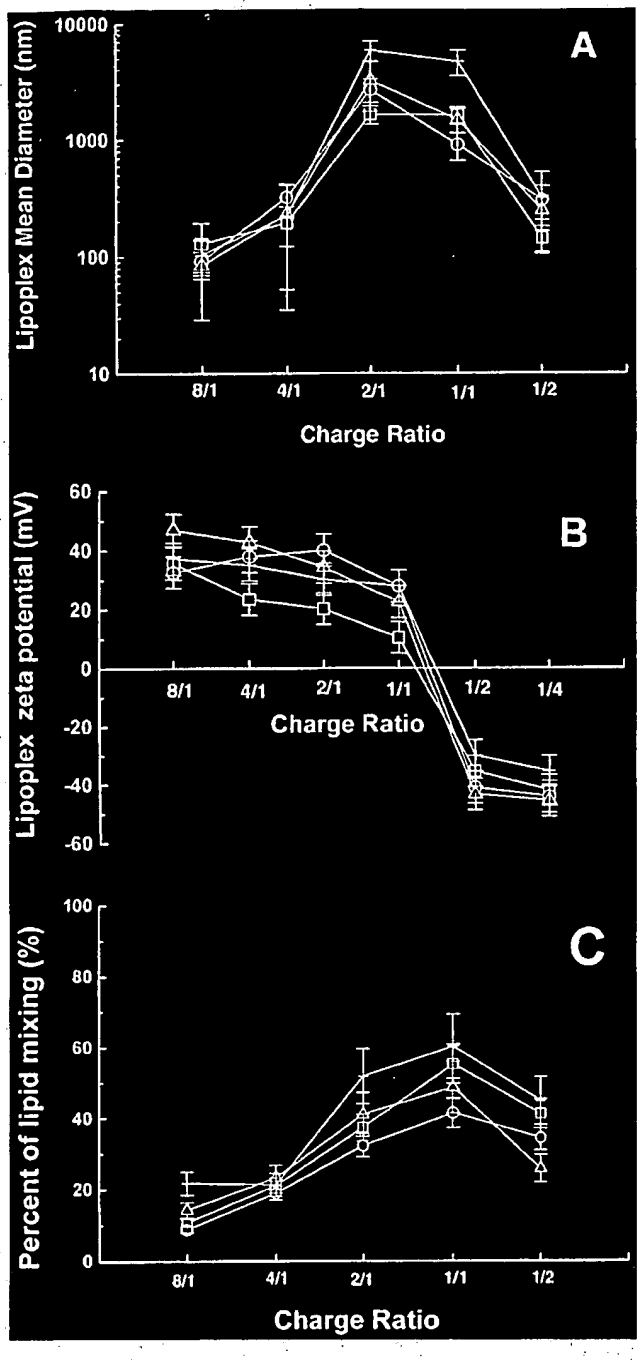

FIGURE 1 Cationic liposome/DNA complex colloidal properties. The symbols represent lipoplexes formed from the following lipid compositions: □, DOTAP; △, DOTAP/DOPE; ○, DOTAP/MOG; +, DOTAP/DOPC. All of the samples were measured at least three times, and the error bar is the standard deviation of these measurements. (*A*) Diameter. The diameters of the starting sonicated liposomes were 40 nm for DOTAP, 10 nm for DOTAP/MOG, 40 nm for DOTAP/DOPE, and 80 nm for DOTAP/DOPC. The complexes were prepared at 100 μg/ml DNA final concentration. (*B*) Zeta potential. The complexes prepared as above were diluted in buffer to a final concentration of 30 mM NaCl for the zeta potential measurements. (*C*) Percentage of lipid mixing. The percentage of lipid mixing was calculated as described in Materials and Methods.

centration was 100 μg/ml. At cationic lipid/DNA charge ratios between 1:1 and 2:1, the complexes have the largest diameter (Fig. 1 *A*). Higher or lower charge ratios result in lipoplexes with smaller diameters. All of the cationic liposomes formed lipoplexes of about the same diameter when compared at each charge ratio, except for compositions containing DOPC, which formed slightly larger diameter lipoplexes at the 2:1 charge ratio (Fig. 1 *A*).

The lipoplex zeta potential changes from negative to positive when the mixing charge ratio changes from excess negative to excess positive. The lipoplexes mixed at 1:1 charge ratio had a positive zeta potential when they were mixed using the standard protocol: injecting DNA into liposome solutions. However, if liposomes were injected into DNA solution at the same charge ratio, the zeta potential of the resulting complexes was negative (Tros de Ilarduya and Szoka, unpublished data). At the various charge ratios, the zeta potentials of the complexes were quite similar among complexes formed from different liposome compositions. However, lipoplexes formed with an excess of DOTAP had a slightly lower zeta potential than the lipoplexes formed using other liposome compositions.

Lipid mixing during formation of the lipoplex

Cationic liposomes underwent extensive lipid mixing during their complexation with DNA (Gershon et al. 1993). Lipid mixing increased as the amount of plasmid DNA added increased, up to a maximum value at a 1:1 charge ratio (Fig. 1 *C*). Injecting the lipid into the DNA at a 1:2 charge ratio resulted in significant lipid mixing, albeit slightly decreased from the value found at the 1:1 charge ratio. Notably, all of the liposome formulations examined exhibited a similar extent of lipid mixing, even in the case of DOTAP/DOPC, in which the presence of DOPC was expected to have an inhibitory effect on membrane fusion. As we show later, the lipid mixing assay does not predict transfection activity.

Displacement of EtBr Intercalation

When EtBr intercalated into DNA, its fluorescence emission at 610 nm was greatly enhanced. Formation of the cationic liposome/DNA complex resulted in the displacement of EtBr, causing a sharp decrease in the fluorescence. The displacement of EtBr from DNA was rapid and was usually completed within 30 s (Xu and Szoka, 1996). With the addition of lipid to the DNA, there was a gradual decrease in fluorescence, until the cationic liposome/DNA charge ratio reached 1:2 (Fig. 2 *A*). At a greater charge ratio, there was a substantial drop in fluorescence, so that at a 2:1 ratio nearly 90% of the EtBr was displaced from DNA. Previously Gershon and co-workers (1993) had shown this phenomenon with a DOTMA/DOPE composition. Eastman and co-workers (1997) have shown that over a narrow range of charge ratios near charge neutrality, the decrease in fluorescence is linear; we looked at a larger range in charge

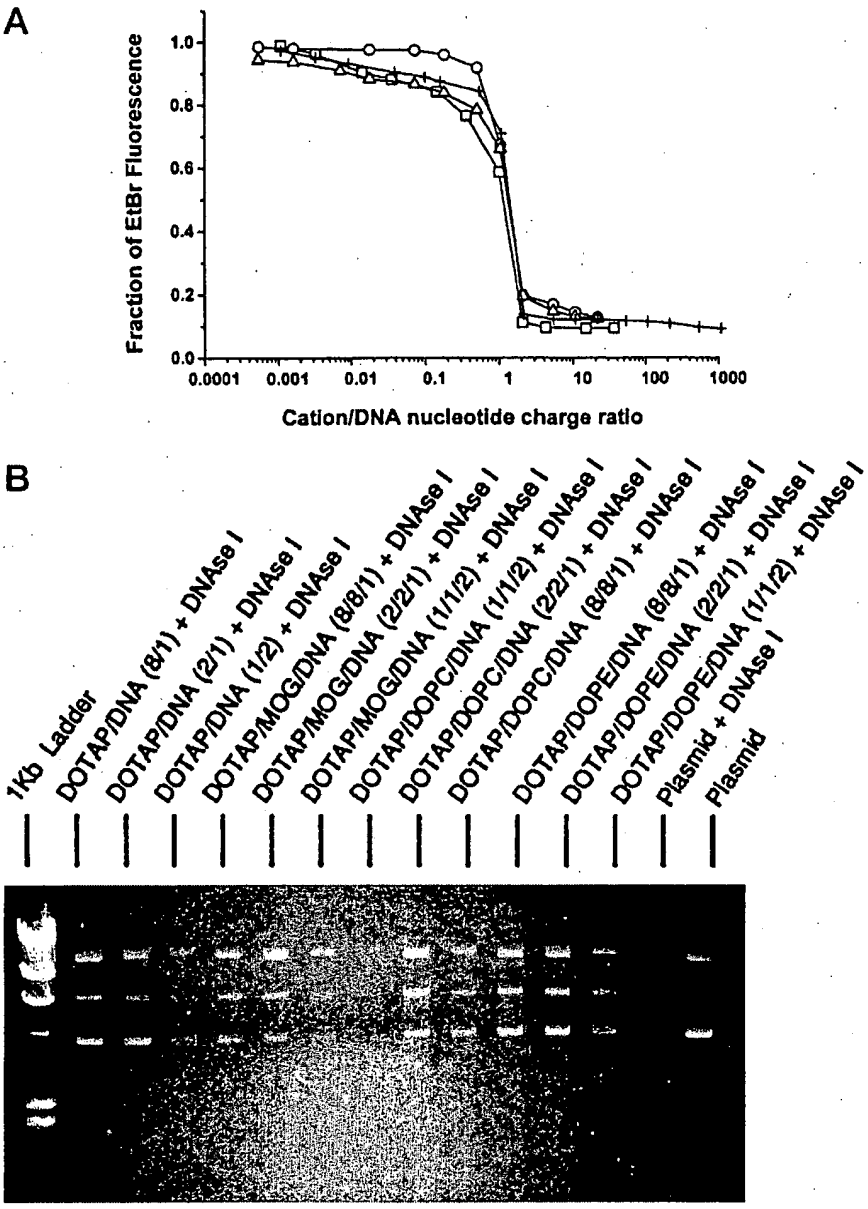

FIGURE 2 Accessibility of plasmid DNA to EtBr intercalation and DNAse I digestion. (*A*) Displacement of EtBr intercalation. Plasmid DNA (5 μg/ml) was incubated with 0.2 μg/ml EtBr, and the fluorescence at 610 nm (excitation 500 nm) was taken as the maximum fluorescence. Various amounts of cationic liposomes were added to the solution, and the change in fluorescence intensity was measured. □, DOTAP; △, DOTAP/DOPE; ○, DOTAP/MOG; +, DOTAP/DOPC. (*B*) DNAse I digestion of cationic liposome/DNA complexes. Cationic liposome/DNA complexes were prepared at the mixing molar ratios indicated in the graph. The complexes were exposed to DNAse I (2 units/μg of DNA) for 30 min in a reaction buffer consisting of 0.9 mM $MnCl_2$, 50 mM Tris-HCl (pH 7.4).

ratios, so the decrease in fluorescence appears to be more abrupt as the positive-to-negative charge ratio is increased. We found that all of the cationic liposome compositions examined (DOTAP, DOTAP/MOG, DOTAP/DOPE, DOTAP/DOPC) displaced EtBr to a similar extent.

Protection against DNase I digestion

After forming complexes with cationic liposomes, DNA molecules were much more resistant to DNase I digestion. Plasmid recovered after 30-min exposure to DNase I is shown in Fig. 2 *B*. Naked plasmid DNA was completely degraded within 5 min, whereas in complexes mixed at 2:1 and 8:1 charge ratios, most of the plasmid DNA was intact after 60 min of DNAse I digestion. Plasmids that were mixed with cationic liposomes at 1/2 charge ratio were partially protected from degradation. Here again, all of the cationic liposome formulations examined protected DNA against DNase I digestion to a similar extent.

Freeze-fracture electron microscopy of cationic lipid/DNA complexes

To see the detailed membrane morphology of the various lipoplexes formed at different charge ratios and with different lipid compositions, we examined the freeze-fractured lipoplex replicas with an electron microscope (Figs. 3 and 4).

We confirmed previous observations (Sternberg et al., 1994) that the membrane morphologies observed in the lipoplex were strikingly different from those observed in the liposomes. Cationic liposomes, after sonication, were mostly unilamellar vesicles with an aqueous interior.

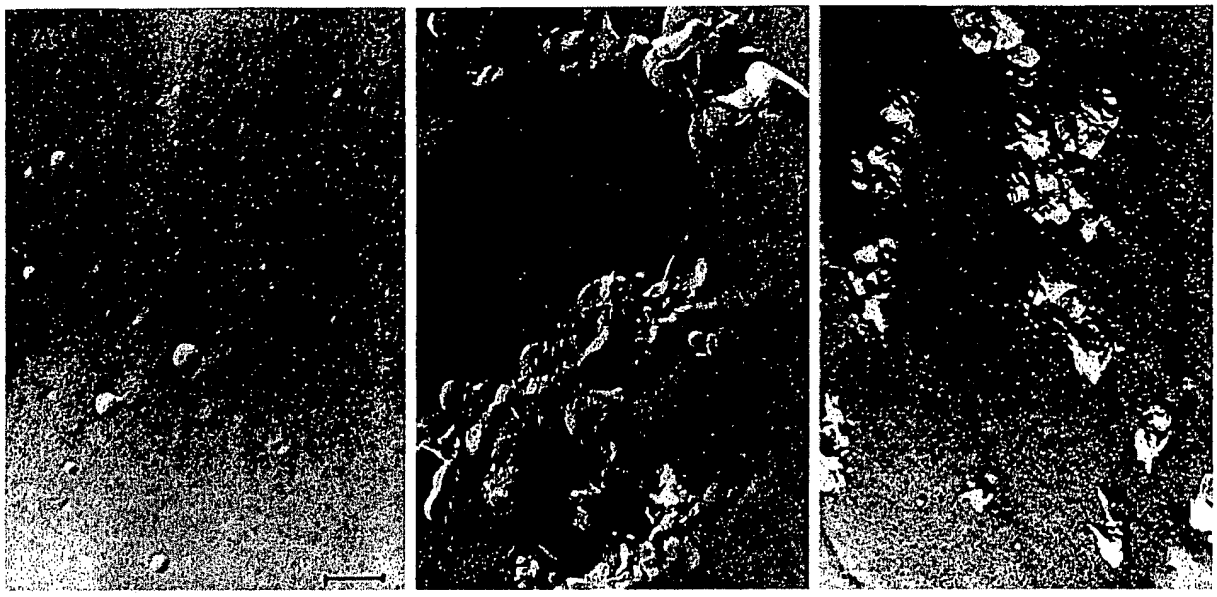

FIGURE 3 Freeze-fracture electron micrographs of DOTAP/DNA at various charge ratios. (*A*) DOTAP unilamellar vesicles. (*B*) DOTAP/DNA complexes formed with excess lipid. (*C*) DOTAP/DNA complexes formed with excess DNA. The bar represents 100 nm.

DOTAP liposomes were between 30 and 60 nm in diameter (Fig. 3 *A*). After forming a complex with DNA in excess lipid, aggregates were observed with partially fused lipid membranes (Fig. 3 *B*). The membranes observed in the aggregates were deformed into numerous, highly curved membrane features with diameters less than 10 nm. When DNA was in excess, the liposome disruption was even more striking (Fig. 3 *C*). The aggregated liposomes were extensively distorted, and the appearance of the fracture planes suggested that the bilayers were no longer vesicular (Fig. 3 *C*). Parallel strands, believed to be lipid-ensheathed DNA, can be discerned on some fracture planes (*arrows*).

An interesting feature of the freeze-fracture images is that when liposomes containing different helper lipids were used to form the lipoplexes, distinctively different features in membrane morphology were observed. DOTAP/DNA complexes had many spike-like strands (Figs. 3 *B* and 4 *A*), DOTAP/MOG/DNA complexes were surrounded by tiny granules (Fig. 4 *B*), and DOTAP/DOPE/DNA complexes contained many elongated tubes (Fig. 4 *C*) and appeared similar to previous reports of complexes formed between a cationic cholesterol/DOPE composition and DNA (Sternberg et al., 1994). Lipoplexes composed of DOTAP/DOPC/DNA had areas of smooth membranes and some spike-like features (Fig. 4 *D*) similar to those observed in DOTAP/DNA complexes.

Purification of DOTAP lipoplexes from components

To obtain a better understanding of the colloidal and structural properties of the lipoplexes, we thought it would be useful to separate lipoplexes from excess components. There is a substantial difference in the densities of liposomes, DNA, and their lipoplexes, so they can be separated from each other by the use of a density gradient. We used DOTAP lipoplexes for the separation study because they were the most active in cell transfection (see below). DOTAP/DNA complexes were formed at different charge ratios and separated by the use of a linear sucrose gradient (0–30%). The fractions at different densities were collected and analyzed for their lipid and DNA content. The profiles of lipid and DNA distribution for complexes prepared at charge ratios of 10:1, 5:1, 2:1, 1:1, 1:2, and 1:5 are shown in Fig. 5. Discrete bands of liposomes, lipoplexes, and DNA molecules were obtained. Liposomes floated on top of the gradient (0–3%), whereas DNA molecules settled to the bottom (>25%) of the gradient. Lipoplexes formed a discrete band in the gradient (10%–20%), the exact position of which was determined by the lipid/DNA ratio in the complex. The lipoplex bands were usually discrete and fell into two categories: at charge ratios greater than 2:1, the complexes migrated to approximately the 11% sucrose concentration region, and the excess liposomes remained at the top of the gradient. At charge ratios greater than 1:1, the complexes sediment to the 18% sucrose concentration region. The greater the DNA excess, the more DNA molecules were found at the bottom of the gradient. The lipoplexes formed at a charge ratio of 1:1 were extensively aggregated after sedimentation. They usually formed an extended sheet of aggregated material that made it difficult to remove them from the gradient and redisperse them in buffer. Occasionally, multiple bands of lipoplex material were observed, especially for complexes prepared at a charge ratio close to 1:1. The minor bands were very faint and varied from

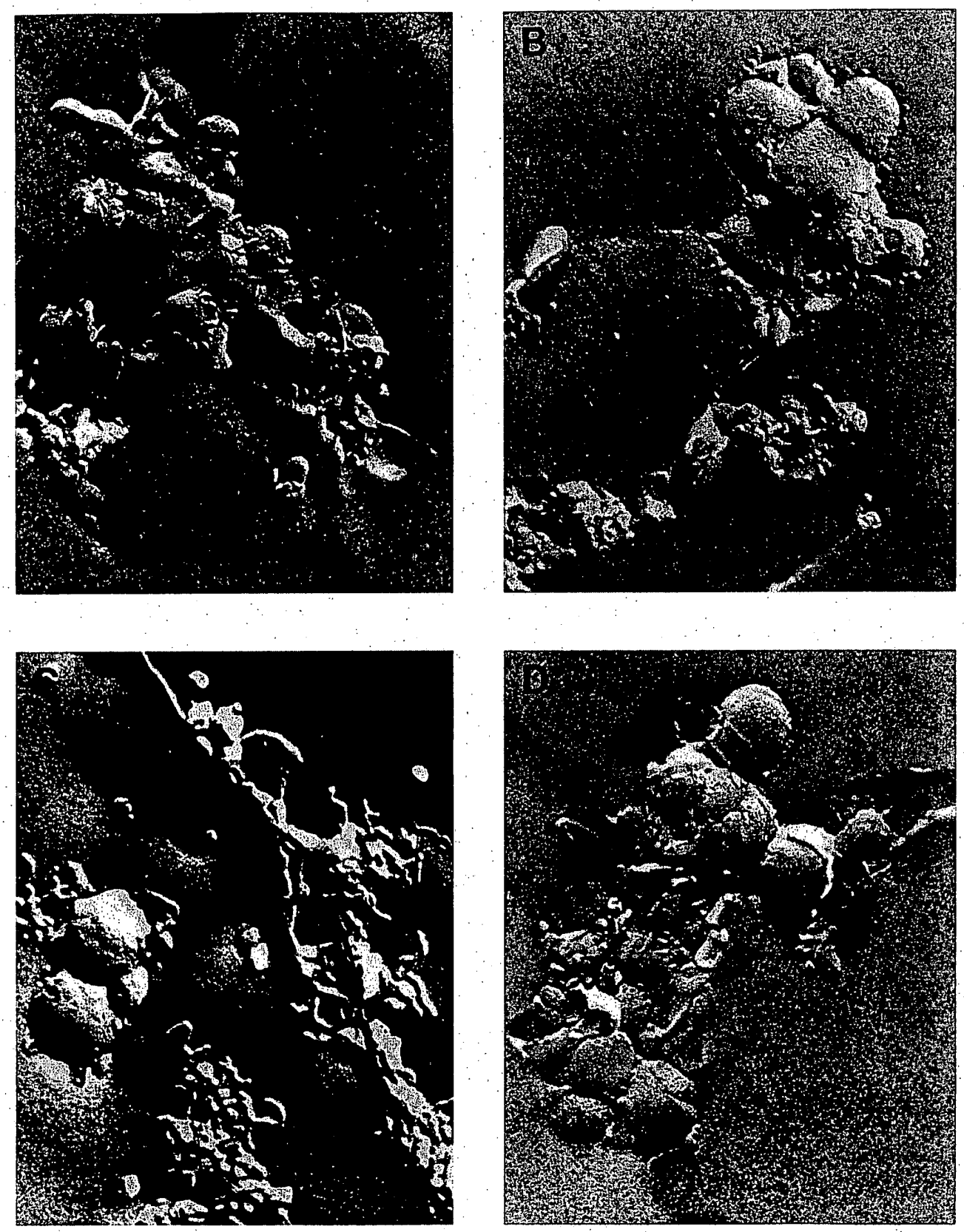

FIGURE 4 Freeze-fracture electron micrographs of cationic liposome/DNA complexes containing different lipid compositions. All of the complexes were prepared by rapidly mixing DNA into the liposomes at a 2:1 charge ratio. (*A*) DOTAP/DNA. (*B*) DOTAP/MOG/DNA. (*C*) DOTAP/DOPE/DNA. (*D*) DOTAP/DOPC/DNA. The bar represents 100 nm.

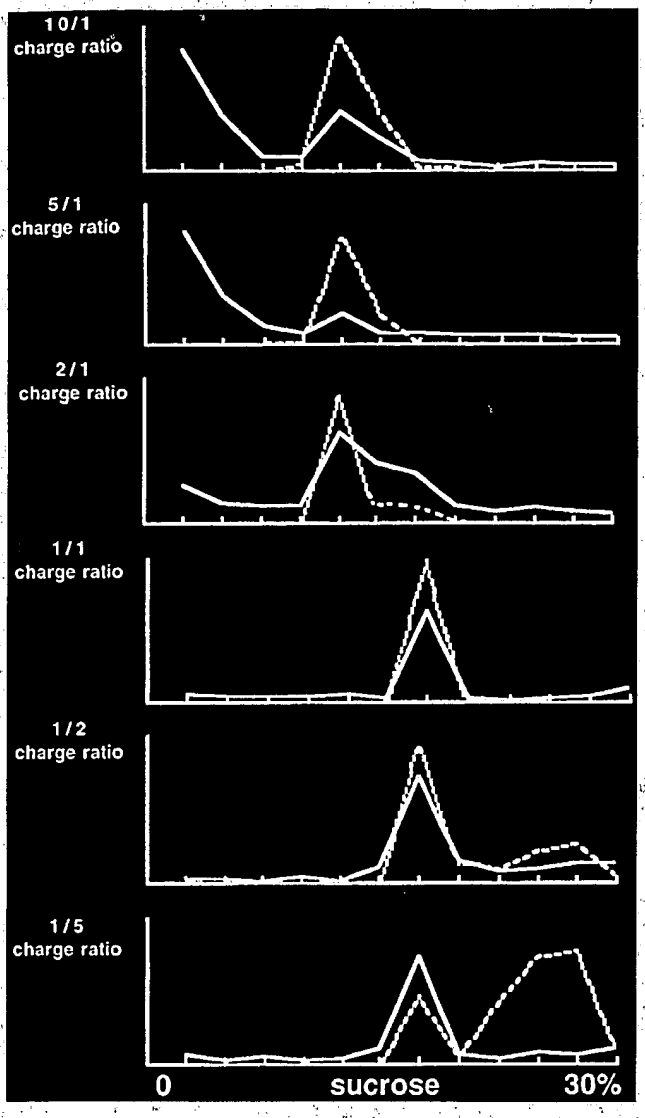

FIGURE 5 Sucrose gradient separation of DOTAP/DNA complexes mixed at different charge ratios. This is a representative data plot from three experiments. The solid line represents the distribution of DOTAP in the gradient. The dashed line represents the distribution of DNA in the gradient. Arbitrary scales are used at the *y* axis for easy presentation. The total recovery of lipid and DNA from all of the fractions is usually >80% of the amount used. Sucrose concentration in the gradient varies linearly from 0% to 30% from left to right. Plotted from top to bottom are DOTAP/DNA complexes mixed at charge ratios of 10:1, 5:1, 2:1, 1:1, 1:2, 1:5.

preparation to preparation. The farther the initial charge ratio was from 1:1, the sharper and more precisely positioned was the lipoplex material.

Characterization of separated lipoplexes

The separated complexes were isolated and characterized for their size distribution, zeta potential, lipid/DNA composition, microscopic structures, and transfection activities.

Diameter

The diameter of the separated complexes is plotted in Fig. 6 *A*. The lipoplex diameters were less than 100 nm when they

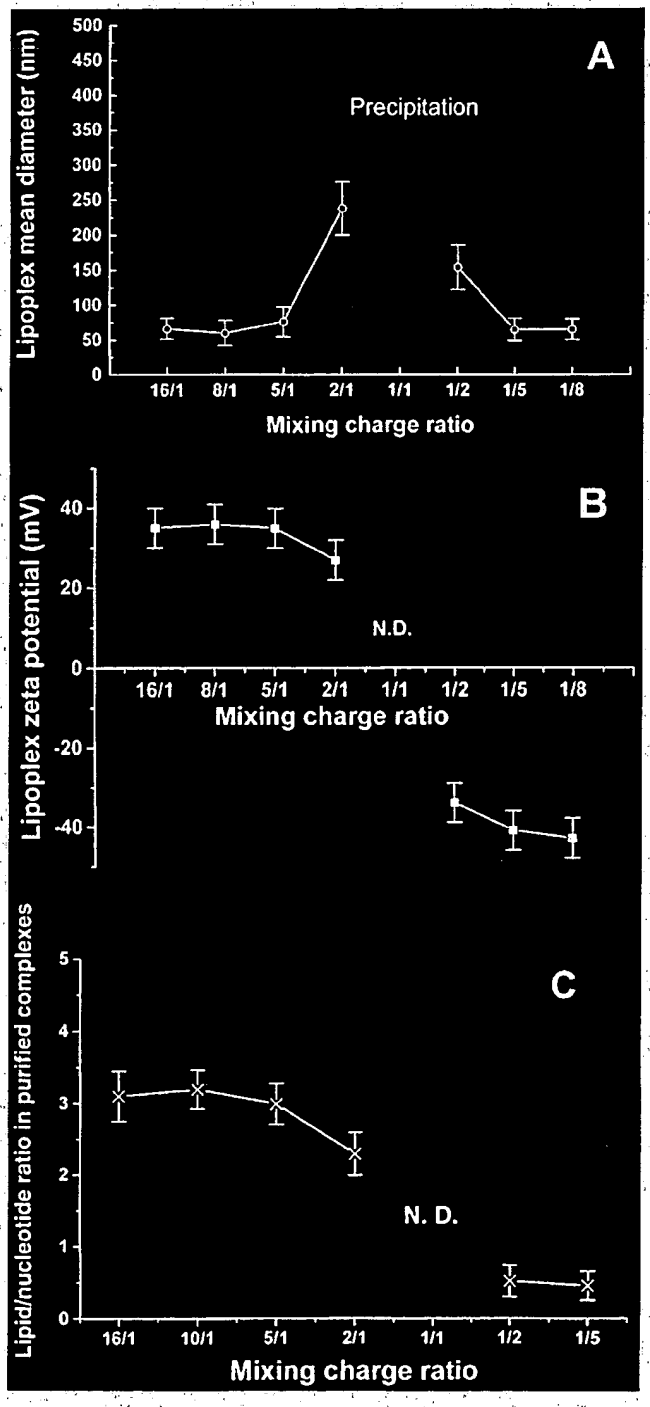

FIGURE 6 Characterization of separated DOTAP/DNA complexes prepared at different charge ratios. The complexes were mixed at the indicated charge ratio and separated by centrifugation on sucrose gradients as described in Materials and Methods. All experiments were repeated three times, and the error bars represent the standard deviation. (*A*) Mean diameter of the separated complexes. (*B*) Zeta potential of the separated complexes. (*C*) DOTAP/DNA stoichiometry in the separated complexes.

were prepared at a large excess of either liposomes or DNA. This is similar to diameters measured on unseparated complexes (Fig. 1 *A*). However, the separated complexes had a more homogenous size distribution (SD = ±20 nm). At a mixing charge ratio close to 1:1, visible aggregates were always observed after separation.

Zeta potential

The zeta potential of the separated complexes usually fell into two groups, regardless of the charge ratios at which they were prepared (Fig. 6 *B*). Those formed with excess cationic liposomes had zeta potentials close to 30 mV, resembling lipoplexes formed at a 2:1–4:1 charge ratio but without separation (Fig. 1 *B*). Lipoplexes formed with excess DNA were negatively charged, with zeta potentials around −40 mV, resembling unseparated lipoplexes formed at 1:2–1:4 charge ratio (Fig. 1 *B*). Thus two types of complexes were found after separation: positively charged and negatively charged lipoplexes. The zeta potential of complexes formed at a 1:1 charge ratio could not be measured because of the formation of large aggregates during the separation.

Stoichiometry

The separated lipoplexes can also be divided into two groups according to their cationic lipid/nucleotide ratios (Fig. 6 *C*). When mixed with excess lipid, the lipid/nucleotide ratio of the separated lipoplex as determined by fluorescence measurements of the DNA and lipid was ~3:1 (Fig. 6 *C*) and did not depend on the initial mixing ratio. When the lipid/DNA ratio was determined using fluorescent lipid and radiolabeled DNA, the ratio was 3.5 ± 0.3:1. When the lipoplex was formed with excess DNA, the resulting ratio, determined from the fluorescence measurement of lipid and DNA, was close to 1:1.8 (Fig. 6 *C*). This was also independent of the initial mixing ratio. When the ratio was determined using fluorescent lipid and radiolabeled DNA, it was 1:2.2 ± 0.2. The two methods for computing the charge ratio of the separated complexes agree to within the precision of the measurements.

Cryoelectron microscopy of the lipoplexes

Examination of the mixtures before separation by cryoelectron microscopy showed occasional compact structures in contact with spherical bilayer vesicles of various diameters (Fig. 7 *A*). The vesicles appeared to be aggregated and were of greater diameter than the starting DOTAP liposomes (Fig. 3 *A*). In contrast, the separated complexes prepared by mixing a large excess of either component showed compacted structures with a much denser interior (Fig. 7, *B* and *C*). The complexes were small and compacted, with very few empty liposomes or free DNA strands visible, compared to the loosely associated liposome aggregates observed before separation. The basic features in the membrane/DNA-rich substructures appeared similar in terms of electron density and substructure before and after separation, suggesting that the basic lipid/DNA association mode was not changed by the gradient process.

Differences between the positive and negative charged complexes are clearly visible. The positive complexes were filled with uniformly dense structures, with some toroid-shaped features visible, whereas the negatively charged complexes had a much more visible periodic pattern of 5.9 ± 0.1 nm consisting of electron-dense strips alternating with relatively electron transparent regions. Because the complexes were viewed as 2D projections, microscopic membrane features such as those observed in freeze-fracture electron micrographs may not be visible. Certain properties of the separated cationic lipid/DNA association structure can still be deduced; the lipid membranes in the complexes were clearly deformed and rearranged as a result of the strong electrostatic interaction between cationic lipid headgroup and DNA phosphate.

In vitro transfection activity

Different lipid formulations were tested for their in vitro transfection activity both before and after separation on the sucrose gradient. Before separation DOTAP had the highest transfection activity at a DNA dose of 0.6 μg/well (Fig. 8 *A*). DOTAP/MOG, DOTAP/Chol, and DOTAP/DOPE had lower but still substantial transfection activities, whereas DOTAP/DOPC was almost inactive in transfection. This is despite the fact that all of the lipoplexes had similar colloidal properties and a similar ability to protect DNA from degradation by DNase I. In all cases the transfection activity peaked at a 2:1–4:1 charge ratio and decreased with higher or lower charge ratios.

Purified lipoplexes showed many advantages compared to the unprocessed mixtures in cell transfection (Fig. 8 *A–C*). The elimination of excess cationic liposomes from purified positively charged complexes extended the nominal charge ratio, where high activity was observed to 16:1 (Fig. 8 *B*). This is probably due to the greatly reduced cytotoxicity from excess cationic liposomes that have been removed from the mixture. Purified negatively charged lipoplexes showed moderate transfection activity compared to the negligible activity of the mixtures prepared at a charge ratio of less than 1:1 and not separated (Fig. 8, *A* and *C*). The activities of these two separated complexes were proportional to the DNA dose used and can be improved by using a higher DNA dose (1.5 μg/well), as shown in Fig. 8, *B* and *C*.

Another interesting property of the separated lipoplexes is that they were much more resistant to the inhibitory effects of serum (Fig. 8, *B* and *C*). When the serum content in the incubation medium used for the transfection was increased to 30%, the negatively charged complex exhibited as high a transfection activity as found with the positively charged complex under the same conditions.

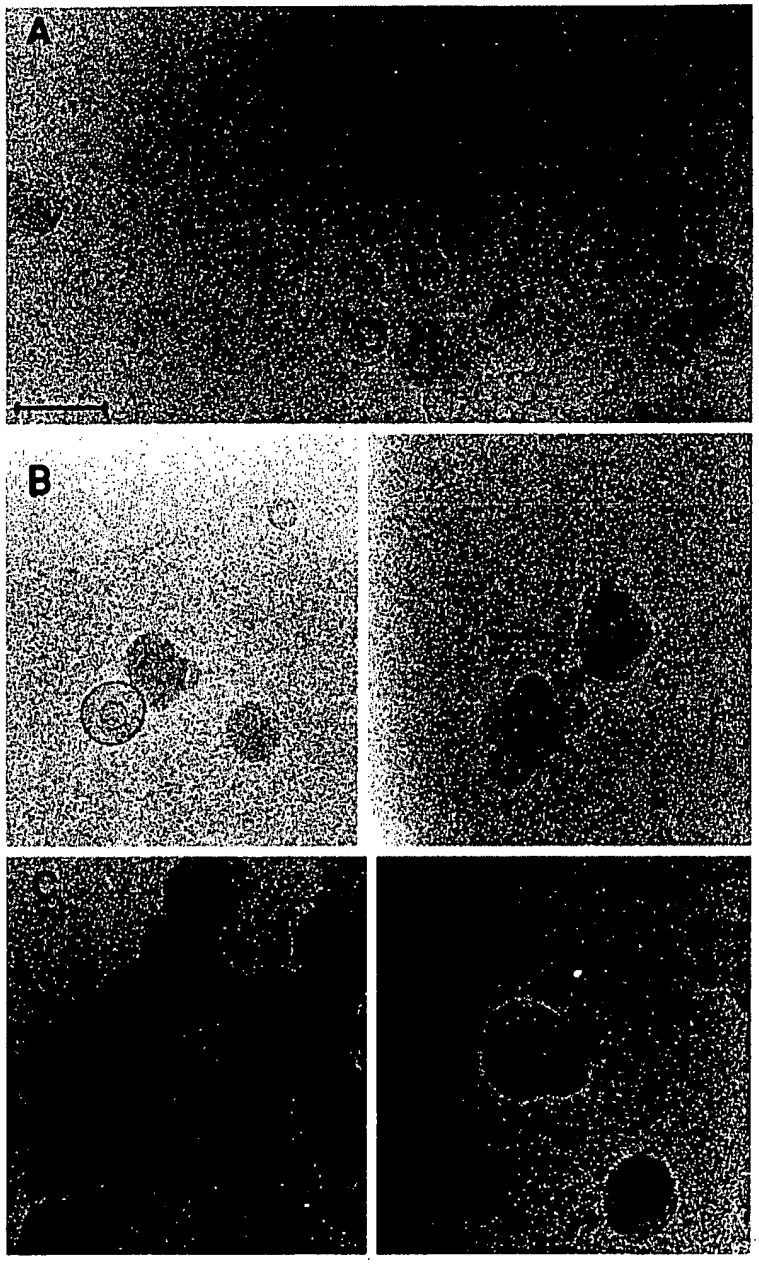

FIGURE 7 Cryoelectron micrographs of separated DOTAP/DNA complexes. (*A*) DOTAP/DNA complexes prepared at a charge ratio of 10:1 before separation. (*B*) DOTAP/DNA complexes prepared at a charge ratio of 10:1 and separated by centrifugation on a linear sucrose gradient. (*C*) DOTAP/DNA complexes prepared at a charge ratio of 1:4 and separated by centrifugation on a sucrose gradient. The bar represents 100 nm.

DISCUSSION

Cationic liposome/DNA lipoplexes are widely used in cell transfection (Felgner et al., 1987) and for in vivo gene delivery (Gao and Huang, 1995). However, the relationship between activity and the physicochemical properties of the lipoplexes is not understood. Gershon and co-workers (1993) were the first to provide a detailed account of the kinetics of lipoplex formation and the structure of the complex formed from DOTMA/DOPE and DNA. These investigators proposed that a threshold ratio of cationic lipid was required to induce collapse of the DNA into a rod-shaped lipidic particle. Upon formation of the complex there was a simultaneous rearrangement of the associated lipid via fusion of the cationic liposomes and DNA collapse. The complex displaced ethidium bromide and protected DNA from degradation from DNase. Based upon these findings, Gershon suggested that the efficient encapsidation process may be responsible for the efficiency of cationic liposome-mediated transfection. Other groups investigated the effect of the ratio of cationic lipid to DNA (Mahato et al., 1995; Eastman et al., 1997; Lasic et al., 1997; Radler et al., 1997) or oligonucleotide (Jaaskelainen et al., 1994) and found similar results in terms of protection of DNA from ethidium bromide intercalation and nucleic acid-induced mixing of the lipid. In addition, they confirmed the expectation that the zeta potential of the lipoplex depends upon the initial charge ratio, and the diameters of the resulting complexes increase and become more polydisperse as the charge equiv-

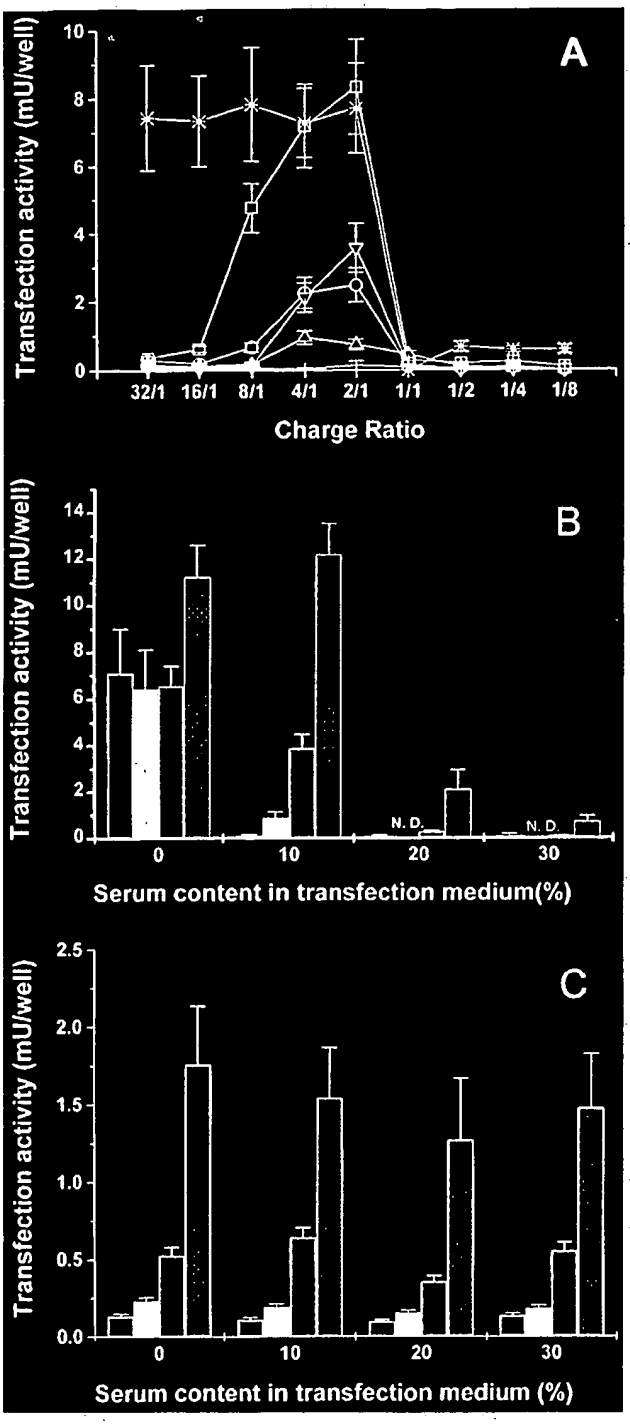

FIGURE 8 Transfection of CV1 cells. (*A*) Transfection using lipoplexes containing various lipid compositions before and after separation. Cationic liposomes were mixed with DNA (24 μg/ml) at the indicated charge ratios, and 0.6 μg of plasmid DNA was used for each well in a 96-well plate. □, DOTAP; △, DOTAP/DOPE; ○, DOTAP/MOG; ▽, DOTAP/Chol; +, DOTAP/DOPC; *, DOTAP after separation. (*B*) Transfection using purified positively charged lipoplexes. Purified positively charged DOTAP/DNA lipoplex, which was prepared at a 16:1 charge ratio and separated from excess liposomes as above, was compared to unpurified lipoplexes prepared at a 4:1 charge ratio. Two DNA doses are plotted: a normal dose (0.6 μg/well) and a high dose (1.5 μg/well). □, Unpurified complex, normal dose. ■, Unpurified complex, high dose. ▨, Purified complex, normal dose. ▩, Purified complex, high dose. (*C*) Transfection using purified negatively charged lipoplexes. Purified negatively charged DOTAP/DNA lipoplexes, prepared at a 1:5 charge ratio and separated from excess DNA, were compared with unpurified lipoplexes prepared at a 1:2 charge ratio. Two DNA doses are plotted: a normal dose (0.6 μg/well) and a high dose (1.5 μg/well). □, Unpurified complex, normal dose. ■, Unpurified complex, high dose. ▨, Purified complex, normal dose. ▩, Purified complex, high dose.

alency point is attained, where precipitation of the complexes is often observed.

We have examined in detail the effect of lipid composition and charge ratio on lipoplex colloidal properties and microscopic structures. Our results on characterization of the DOTAP-DNA lipoplexes indicate inclusion of the helper lipids: cholesterol, MOG, DOPE, or DOPC results in lipoplexes that behave in a very similar fashion in terms of their colloidal properties, membrane mixing, and protection of DNA from degradation by DNase or intercalation by ethidium bromide.

Despite the similarities of the colloidal properties, there was a clear distinction in the morphologies observed in freeze-fracture electron micrographs of the various compositions. All lipoplexes show worm-like features partially adsorbed and extended from the surfaces of fused vesicles. These worm-like structures or tubes have a common diameter of 10 nm and are believed to be DNA strands ensheathed in lipid bilayers (Sternberg et al., 1994; Hui et al., 1997). The tubes are extremely short and appear bead-like in lipoplexes containing DOTAP/MOG (Fig. 4 *B*), slightly longer in those containing DOTAP or DOTAP/PC (Fig. 4, *A* and *D*), and only extensively elongated in DOTAP/DOPE lipoplexes (Fig. 4 *C*). The absence of elongated tubes in lipoplex structures formed from cationic lipids different from those studied here was also reported by Eastman and co-workers (1997). The differences in appearance may depend on how readily the lipids in the lipoplexes can phase-separate and form high-curvature sheaths around DNA.

Although each lipid mixture had a characteristic appearance (Figs. 3 and 4), we were unable to identify any obvious structural feature in the images that correlated with the in vitro transfection activity rank order DOTAP ≫ DOTAP/MOG = DOTAP/cholesterol > DOTAP/DOPE ≫ DOTAP/DOPC (Fig. 8). Extensively elongated tubular structures were only observed in lipid mixtures that contained DOPE, which is similar to the report of Sternberg and co-workers (1994); however, our findings indicate that such elongated tubular structures are not required for high transfection activity. There was a tendency of the transfection-active lipid mixtures to have a greater incidence of high-radius structures in the fracture plane than observed in structures formed from the transfection-inactive DOTAP/DOPC mixture. Freeze fractures of lipoplexes prepared at a large excess of lipid contained fracture planes indicative of bilayer sheets that were probably from lipid not associated with the complex. These were absent from the jagged fracture planes of complexes prepared with an excess of DNA.

Thus it appears that all of the lipid compositions studied provide efficient encapsidation, so that the high transfection efficacy of certain compositions must be due to other factors (Xu and Szoka, 1996; Zelphati and Szoka, 1996; Hui et al., 1996).

Because DOTAP/DNA lipoplexes have the highest transfection activity, we studied these lipoplexes in more detail. After purification from the excess component, there are two distinct lipoplexes, those formed with either excess lipid or excess DNA. They have a defined lipid/DNA stoichiometry of 3.5:1 or 1:2.2, respectively. Their diameters are ~70 nm, and their zeta potentials are, respectively, +35 mV and −40 mV. The positively charged lipoplexes appear to have a dense but diffuse interior, together with a few toroid-shaped features (Fig. 7 *B*). The 3.5:1 ratio (±) of cationic lipid to nucleotide raises an interesting question of how the cationic lipid is packed in this structure. It seems that the lipoplex prepared at the higher positive charge ratio might correspond to the situation described by Radler and colleagues (1997), in which the lipid is in excess and the DNA lattice is expanded. Fluctuations in the bilayer separation might then make it difficult to visualize a discrete repeat in the cryoelectron micrographs, accounting for the diffuse dense appearance of the lipoplexes prepared at a high positive charge ratio (Fig. 7 *B*). It is also possible that the charge neutralization of DNA results in DNA bending into donut-like or even spherical structures, as seen in Fig. 7 *B*. The particles are predominantly 70 nm in diameter. This is similar to dimensions of toroids formed between DNA and polycations (Bloomfield, 1996). It may be that the dimensions of these particles are determined by the persistence length of the DNA.

The negatively charged lipoplexes, on the other hand, contain a prominent striated structure with a spacing of 5.9 ± 0.1 nm, as revealed by cryoelectron microscopy (Fig. 7 *C*). The alternative light and dark bands could represent parallel DNA strands sandwiched between lipid bilayers. These images contain structures similar to those published by Gustafasson and colleagues (1995) for unpurified DOTAP/DNA. Freeze-fractured DOTAP/DNA lipoplexes formed in excess DNA also show short parallel strands with a periodicity of 7–8 nm (including the thickness of the carbon/platinum replica) in similar size complexes (Fig. 3 *C*). Both cryoelectron micrographs and freeze-fracture micrographs show loose ends, presumed to be DNA, extending from the edges of lipoplexes. The ordered strands may represent ordered arrays of DNA confined between lipid layers by the limited amount of lipids available. Theoretical models of DNA associated with membranes suggest precisely such behavior, that is, ordered DNA domains should arise when DNA is absorbed to membranes (Dan, 1996). This form of crystalline array has been suggested in the x-ray diffraction models, which revealed a repeating spacing of 5.8 ± 0.2 nm, based on results of lipoplexes with a 1:1 charge ratio (Radler et al., 1997; Lasic et al., 1997). We note that the negatively charged lipoplex has a lipid/DNA packing structure distinctly different from that of the positively charged complex. Surprisingly, it is also transfection active, albeit at a moderate level. So it is less likely that one specific lipid/DNA packing structure is responsible for all of the transfection activity reported for the various cationic lipoplexes.

The transfection activities of both types of purified lipoplexes are independent of their starting charge ratios, with similar activities for positively charged complexes and negatively charged complexes. The transfection activity of the purified positively charged lipoplexes is high (>10 mU/well) and increases when the DNA dose is increased. The separated complexes are much less toxic to the cells compared to the initial mixture. They are also more resistant to the damaging effect of serum, although when serum content reaches 30% the transfection activity is greatly reduced to a residue value (<2 mU/well). Similar observations were reported by Hofland and co-workers, who used a resuspended lipoplex pellet prepared by detergent dialysis (Hofland et al., 1996).

An important finding is that the separated negatively charged lipoplexes have moderate transfection activity compared to the negligible value before separation, which is also independent of serum content (Fig. 8 *C*). The higher activity of the positively charged lipoplexes may be related to their electrostatic attraction to the generally negatively charged cell surfaces (Felgner et al., 1987). The presence of serum apparently inhibits this mode of interaction, either by inhibiting their attachment to the cell surface, or by inhibiting their self-association on the cell surface and eventual internalization by the cell (Sternberg et al., 1994; Hui et al., 1996). However, both positively and negatively charged lipoplexes have a residue transfection activity, which may be based on events not affected by the charge of the lipoplexes (such as random collision and spontaneous fusion with the cell surface).

The finding that the transfection activities of the separated complexes are more resistant to the effects of serum than unseparated lipoplexes is very promising for in vivo gene therapy and is another approach (Hofland et al., 1996; Sternberg et al., 1998) for creating cationic lipoplexes that are stable in serum. The separated complexes are small, homogeneous, and relatively stable upon storage (data not shown). The moderate but serum-resistant transfection activity of the separated negatively charged complexes may be useful for in vivo applications.

SUMMARY

We show that the colloidal properties of lipoplexes are determined by the charge ratio of the DNA and cationic lipid and are not appreciably affected by the type of helper lipid. The lipoplexes have membrane features characteristic of their lipid composition, but no obvious correlation was found between any specific membrane feature and the transfection activity. Finally, lipoplexes of defined stoichiometries can be separated from precursors by sedimentation of

the mixture to equilibrium on sucrose gradients. The separated lipoplexes have a small particle diameter with a high radius of curvature. Lipoplexes with a positive zeta potential differ in structure from those with a negative zeta potential when viewed by cryoelectron microscopy. These separated lipoplexes are resistant to the inhibitory effect of serum in in vitro transfection. The defined structure should prove useful in determining the factors that are critical to transfection activities in cells and animals.

We thank members of the Szoka group for critical reading of the manuscript and their many helpful suggestions.

Funding for this project was provided by the National Institutes of Health (DK46052 to FCS and GM30969 to S-WH), the State of California Tobacco Research Program (6RT-0109 to FCS), and the Gene Therapy Core Center, National Institutes of Health (DK47766 to FCS).

REFERENCES

Bloomfield, V. A. 1996. DNA condensation. *Curr. Opin. Struct. Biol.* 6:334–341.

Dan, N. 1996. Formation of ordered domains in membrane-bound DNA. *Biophys. J.* 71:1267–1272.

Duzgunes, N., J. A. Goldstein, D. S. Friend, and P. Felgner. 1989. Fusion of liposomes containing a novel cationic lipid, *N*-[2,3-(dioleyloxy)propyl]-*N,N,N*-trimethylammonium: induction by multivalent anions and asymmetric fusion with acidic phospholipid vesicles. *Biochemistry.* 28:9179–9184.

Eastman, S. J., C. Siegel, J. Tousignant, A. E. Smith, S. H. Cheng, and R. K. Scheule. 1997. Biophysical characterization of cationic lipid:DNA complexes. *Biochim. Biophys. Acta.* 1325:41–62.

Farhood, H., N. Serbina, and L. Huang. 1995. The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. *Biochim. Biophys. Acta.* 1235:289–295.

Felgner, P. L., Y. Barenholz, J. P. Behr, S. H. Cheng, P. Cullis, L. Huang, J. A. Jessee, L. Seymour, F. C. Szoka, and A. R. Thierry. 1997. Nomenclature for synthetic gene delivery systems (editorial). *Hum. Gene Ther.* 8:511–512.

Felgner, P. L., T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, and J. P. Northrop. 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA.* 84: 7413–7417.

Felgner, J. H., R. Kumar, C. N. Sridhar, C. J. Wheeler, Y. J. Tsai, R. Border, P. Ramsey, M. Martin, and P. L. Felgner. 1994. Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. *J. Biol. Chem.* 269:2550–2561.

Frederik, P. M., K. N. Burger, M. C. Stuart, and A. J. Verkleij. 1991. Lipid polymorphism as observed by cryo-electron microscopy. *Biochim. Biophys. Acta.* 1062:133–141.

Gao, X., and L. Huang. 1991. A novel cationic liposome reagent for efficient transfection of mammalian cells. *Biochem. Biophys. Res. Commun.* 179:280–285.

Gao, X., and L. Huang. 1995. Cationic liposome-mediated gene transfer. *Gene Ther.* 2:710–722.

Gershon, H., R. Ghirlando, S. B. Guttman, and A. Minsky. 1993. Mode of formation and structural features of DNA-cationic liposome complexes used for transfection. *Biochemistry.* 32:7143–7151.

Gustafsson, J., G. Arvidson, G. Karlsson, and M. Almgren. 1995. Complexes between cationic liposomes and DNA visualized by cryo-TEM. *Biochim. Biophys. Acta.* 1235:305–312.

Hofland, H. E. J., L. Shephard, and S. Sullivan. 1996. Formation of stable cationic lipid/DNA complexes for gene transfer. *Proc. Natl. Acad. Sci. USA.* 93:7305–7309.

Hui, S. W. 1988. Freeze fracture studies of membrane lipids. *Electron Microsc. Rev.* 1:351–368.

Hui, S. W., M. Langner, Y. L. Zhao, P. Ross, E. Hurley, and K. Chan. 1996. The role of helper lipids in cationic liposome mediated gene transfer. *Biophys. J.* 71:590–599.

Hui, S. W., Y. L. Zhao, E. Hurley, and P. Ross, P. 1997. The uptake pathway of DNA and lipids in cationic liposome-mediated gene transfer. *In* Focus on Modern Microscopy. J. L. Wu and P. C. Cheng, editors. World Science Publishing, Singapore.

Jaaskelainen, I., J. Monkkonen, and A. Urtti. 1994. Oligonucleotide-cationic liposome interactions. A physicochemical study. *Biochim. Biophys. Acta.* 1195:115–123.

Keren-Zur, M., M. Beigel, and A. Loyter. 1989. Induction of fusion in aggregated and nonaggregated liposomes bearing cationic detergents. *Biochim. Biophys. Acta.* 983:253–258.

Lasic, D. D., H. Strey, M. C. Stuart, R. Podgornik, and P. M. Frederik. 1997. The structure of DNA-liposome complexes. *J. Am. Chem. Soc.* 119:832–833.

Legendre, J. Y., and F. C. Szoka. 1992. Delivery of plasmid DNA into mammalian cell lines using pH-sensitive liposomes: comparison with cationic liposomes. *Pharm. Res.* 9:1235–1242.

Leventis, R., and J. R. Silvius. 1990. Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles. *Biochim. Biophys. Acta.* 1023:124–132.

Mahato, R. I., K. Kawabata, T. Nomura, Y. Takakura, and M. Hashida. 1995. Physicochemical and pharmacokinetic characteristics of plasmid DNA/cationic liposome complexes. *J. Pharm. Sci.* 84:1267–1271.

McGregor, G. R., and C. T. Caskey. 1989. Construction of plasmids that express *E. coli* β-galactosidase in mammalian cells. *Nucleic Acids Res.* 17:2365–2370.

Radler, J. O., I. Koltover, T. Salditt, and C. R. Safinya. 1997. Structure of DNA-cationic liposome complexes: DNA intercalation in multilamellar membranes in distinct interhelical packing regimes. *Science.* 275: 810–814.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning, A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Stamatatos, L., R. Leventis, M. J. Zukermann, and J. R. Silvius. 1988. Interactions of cationic lipid vesicles with negatively charged phospholipid vesicles and biological membranes. *Biochemistry.* 27:3917–3925.

Sternberg, B., K. Hong, W. Zheng, and D. Papahadjopoulos. 1998. Ultrastructural characterization of cationic liposome-DNA complexes showing enhanced stability in serum and high transfection activity in vivo. *Biochim. Biophys. Acta.* 1375:23–35.

Sternberg, B., F. L. Sorgi, and L. Huang. 1994. New structures in complex formation between DNA and cationic liposomes visualized by freeze-fracture electron microscopy. *FEBS Lett.* 356:361–366.

Stuck, D. K., D. Hoekstra, and R. E. Pagano. 1981. Use of resonance energy transfer to monitor membrane fusion. *Biochemistry.* 20: 4093–4099.

Tang, M. X., C. Redemann, and F. C. Szoka, Jr. 1996. In vitro gene delivery by degraded polyamidoamine dendrimers. *Bioconj. Chem.* 7:703–714.

Xu, Y., and F. C. Szoka, Jr. 1996. Mechanism of DNA release in cationic liposome mediated gene transfection. *Biochemistry.* 35:5616–5623.

Zelphati, O., and F. C. Szoka, Jr. 1996. Mechanism of oligonucleotide release from cationic liposomes. *Proc. Natl. Acad. Sci. USA.* 93: 11493–11498.

The use of synthetic polymers for delivery of DNA

Baubak Bajoghli *

* Matrikelnummer: 9802743, Studienkennzahl: A437, E-mail Adresse: a9802743@unet.univie.ac.at

Abstract:

The safe and efficient delivery of therapeutic DNA to cells represents a fundamental obstacle to the clinical success of gene therapy [1,2]. The challenges facing synthetic delivery vectors are particularly clear, as both cationic polymers and liposomes are less effective at mediating gene transfer than viral vectors. The incorporation of new design criteria has led to recent advances toward functional delivery systems [3,4]. However, the paradigm for the development of polymeric gene delivery vectors remains the incorporation of these designelements into materials as part of an iterative, linear process an effective, albeit slow, approach to the discovery of new vectors [1]. The limitations of viral vectors, in particular their relatively small capacity for therapeutic DNA, safety concerns, difficulty in targeting to specific cell types have led to the evaluation and development of alternative vectors based on synthetic, non-viral systems. The main alternatives to viruses, such as polymers, are described below and include liposomes, liposome-polycation complexes and peptide delivery systems. How the synthesis of this polymers are, is not here discussed but for better describtion of DNA delivery process with Polymers, the use of poly L-Lysin for gene delivery in hepatocyte cells are short explained.

Introduction:

In principle, two basic carrier systems, viral (adeno- and retrovirus) and notviral for DNA delivery in target cells are under development. Viruses are the most wiedly used vectors for systemic delivery of genes, but their efficiency of transfection *in vitro* is not reproduced *in vivo* because of their inherent inflammatory properties, coupled with inappropriate tropisms, which restrict their access to target tissues [5]. Several major problems are associated with the use of viral vectors in clinical treatment. The concept of using block or graft copolymers of cationic and hydrophobic nonionic monomers has been introduced as a potential means for development of nonviral gene delivery vectors [6]. The technical challenge is that DNA (commonly a plasmid) is a particulate material with a net negative charge, a negatively charged surface, and a hydrodynamic diameter of >100 nm. This particle must be introduced into the body and delivered to the target cell across various biological barriers, many of which are normally impeneterable by a particle with these characteristics.

Cationic liposomes and polymers have been accepted as effective non-viral vectors for gene delivery with low immunogenicity unlike viral vectors. The main advantage of using cationic polymers is that polymers carrying different structure elements can be developed by self-assembling with DNA by electrostatic interaction to produce vectors with a range of properties.

The development of self-assembling vectors for DNA delivery is based on synthetic block copolymers. The copolymers will be designed to fulfil a number of biological functions, including condensing the DNA in to discrete particles and stabilising it by enshrouding it

within a hydrophilic polymer coating. An important issue for DNA delivery from polymers is what polymer formulations will give an efficient incorporation and a sustained local delivery in a reproducible manner [12].
In non-viral DNA delivery is important that no single formulation can be used to target all somatic targets. Instead, formulations need to be optimized for each somatic target on the basis of the physiological and biological characteristics of that target.

Liposomal gene delivery:

The structural and phase transformations of lipids are generally inherent to nature and can be applied in practice. Negatively charged, or classical, liposomes have been used to deliver encapsulated drugs for some time and have also been used as vehicles for gene transfer into cells in culture. Problems with the efficiency of nucleic acid encapsulation, coupled with a requirement to separate the DNA-liposome complexes from "ghost" vesicles has lead to the development of positively charged liposomes. Cationic lipids are able to interact spontaneously with negatively charged DNA to form clusters of aggregated vesicles along the nucleic acid. At a critical liposome density the DNA is condensed and becomes encapsulated within a lipid bilayer, although there is also some evidence that cationic liposomes do not actually encapsulate the DNA, but instead bind along the surface of the DNA, maintaining its original size and shape.
Since the cellular membrane is negatively charged, it was the cationic polymers that have received the most attention as potential carriers for DNA how antisense Oligondeoxynucleoic. The coulombic forces governing the interaction betwenn plasma membrane and polycations are so strong that the influence of other properties of the polymers (e.g. hydrophilicity) is drastically diminished. However, the cationic polymers do not constitute the ideal solution for antisense Oligode-oxynucleic [8].

Cationic lipids:

The use of lipids with a polar head group (protonated at physiological pH-cationic) as DNA carriers, poineerede in 1987, has resulted in the commercial production of cell cultur gene-delivery kits and the use of lipopsomal gene delivery in clinical trails. A large number of cationic lipids have been synthesised for gene delivery, some of which are shown in Figure 1. All cationic lipids possess an amine group in addition to hydrophobic group. The amino group binds DNA electrostatically, while the hydrophobic groups facilitate the assembly of cationic lipids into bi-layer vesicles. Lipoplexes (cationic liposome/DNA complexes) range from 50 nm to just over 1 μm in size and an increase in lipoplex size improves transfection *in vitro*. A positively charged lipoplex is necessary for cell binding prior to internalisation by endocytosis. Endosomal escape for onward transport to the nucleus is aided by lipids such 1.2-dioleoyl phosphatidylethanolamine (DOPE).
Cationic lipids have also been shown to be effective agents for DNA transfer *in vivo*. Several different cationic lipids have been used to deliver genes to the lung by intratracheal, intravenous (IV), or intrapul-monary artery routes, resulting in efficient
expression of recombinant genes. Gene delivery with cationic liposomes increases the level of gene expression on IV as the lipoplex prevent plasma degradation of DNA. However, these carriers are severely limited in their applicability via the IV route as they are rapidly cleared from the plasma and gene expression occurs primarily in the lung endothelium, the first capillary bed encountered. Expression is transient, peaking between 4 and 24 hours after dosing and disappearing within a weak.

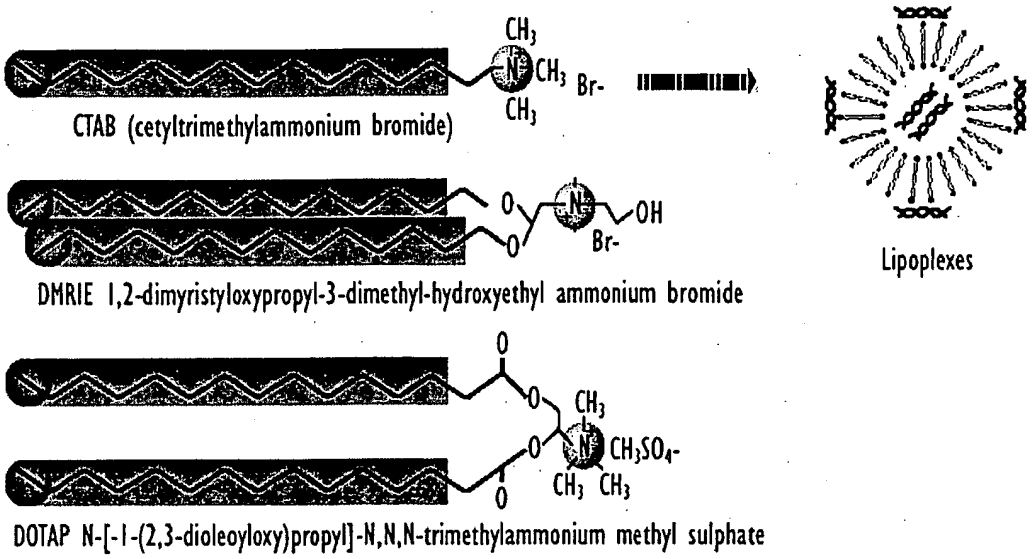

Figure 1: Example of cationic lipids used for DNA delivery

Polymers

Another method for gene delivery utilizes a cationic polymer known as a dendrimer. Dendrimer-DNA complexes are formed by ionic interactions between the positively charged dendrimer and negatively charged DNA. Complexes of different size can be made using dendrimers of different size and different charge ratios. These complexes have been shown to effect efficient gene delivery into a variety of cell types *in vitro* [15].

As with cationic lipids, polymers bearing groups that are protonated at physiological pH have been employed as gene carriers (see Figure 2). The electrostatic attraction between the cationic charge on the polymer and the negatively charged DNA results in a polyplex.

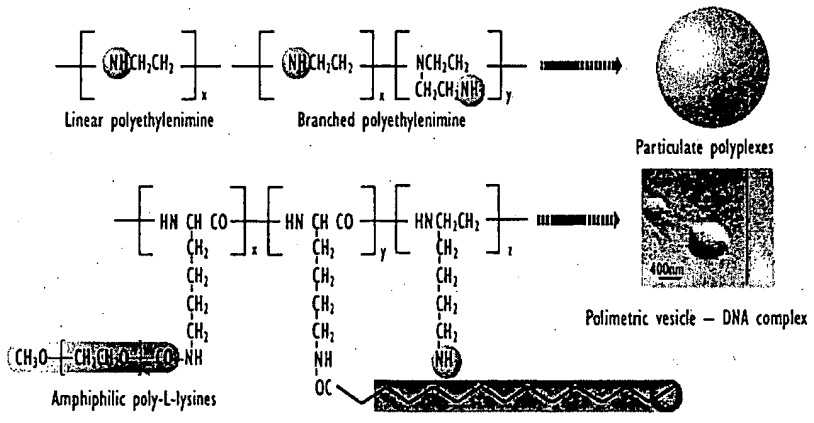

Figure 2: Examples of Cationic Polymers Used for Gene Delivery

Poly L-Lysin (PLL): The use of PLL (Fig 4.a) for gene delivery employs targeting ligands – e.g., asialooroso-mucoid, transferrin and folate, to facilitate receptor – mediated uptake. Without the use of either targeting ligands or lysosomotropic agents (such as chloroquine),

gene transfer is poor with PLL polyplexes alone, an important difference in thebiological activity of the amphiphilic cationic lipids and the soluble polymer PLL. Lipidic PLLs have been prepared and complexed with DNA and the resultant complex was a more efficient transfection agent than cationic liposomes *in vitro*. It appears that amphiphilicity aids gene transfer in some instances. A positively charged polyplex is necessary, presumably to allow interaction with the negatively charged cell surface/endosomolytic uptake, and the incorporation of histidine into PLL polyplexes aids endosomal escape of these agents. A further interesting method of preparing PLL polyplexes involves the replacement of some L-lysine residues with cysteine residues followed by cross-linking to give a superior transfecting unit in which DNA release appears to be triggered by the intracellular reduction of disulphide bonds. Although PLL polyplexes prevent the degradation of DNA by serum nucleases, just like lipoplexes, they are rapidly cleared on IV injection.

From a pharmacokinetic point of view, PLL seems to be a promising hepatocyte-specific carrier; since the hepatotropic nature of cationic polymers is likely due to their positive charge; neutralization of this charge abolishes its affinity for the cells [13]. Complex formation with nucleic acids like plasmid DNA reduces the positive charge of PLL. This, in turn, makes the recognition of glycosylated PLLs via receptors more evident. In addition, the internalization of galactosylated carriers is much faster than that of cationic ones, which could be an advantage for DNA and some drugs [9].

Hepatocyte-specific delivery of DNA was successfully achieved by selecting the most suitable size of PLL and by controlling the galactose density on PLL. If a short PLL with a molecular weight of 1800 is used, a larger amount of PLL is required for complex formation than with PLLs with molecular weights of 13 000 and 29 000. In addition, there is hardly any DNA condensation by the short PLL [13]. To improve the efficiency of galactosylated cationic poly (amino acids)-based gene transfer, the use of compounds that can enhance gene expression such as viruses or viral proteins, fusogenic lipids, and membranedisruptive peptides can be considered. Mitsuru Hashida and his colleagues has suggested that galactosylated polymeric carrier conjugated with a fusogenic peptide was very effective in improving the level of gene transfer after intravenous injection of DNA carrier complexes [17]. Figure 3 gives conceptual explanation about hepatocyte-specific gene delivery with multi-functional polymeric carriers [13].

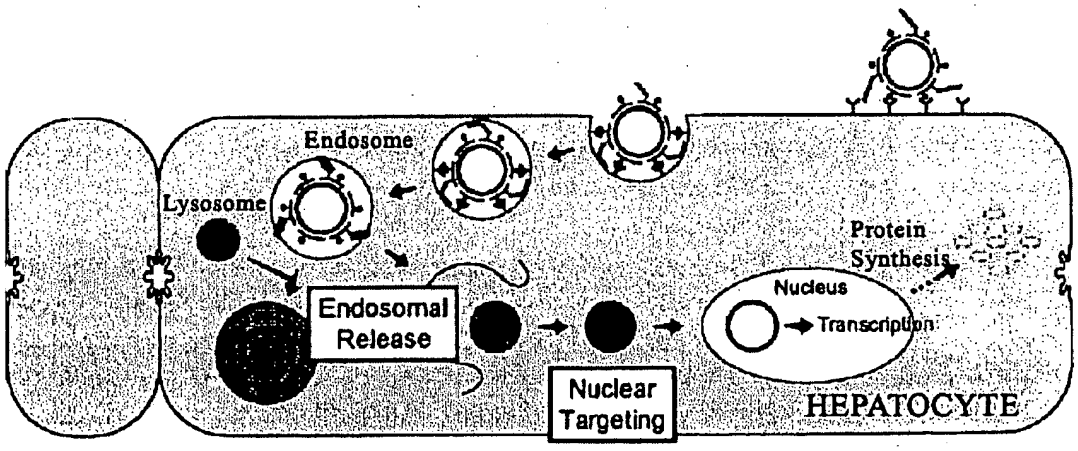

Figure 3: The performance *in vivo* of multifunctional polymeric carriers with galactose moiety for hepatocyte-specific gene delivery [13].

Polyelectrolyte complexes (PECs) are capable of releasing most of the functions mentioned above being, at the same time, suitable for large-scale production. The supramolecular structure of PECs can be described as a hydrophobic core formed by DNA the charge of which was compensated by polycation blocks, surrounded by a shell of hydrophilic polymer chains. So far, several polycations have been used in combination with hydrophilic nonionic polymers to produce complexes with DNA [7].

Polyethylenimine (PEI) is a cationic polymer capable of delivering DNA molecules into cultured mammalian cells as charge complexes (Fig.4.b). The application of PEI polyplexes in gene therapy, however, is hampered by the sensitivity of is transfection activity to the presence of serum.

Both branched and linear PEI shows efficient gene transfer without the need for endosomolytic, lysosomotropic or targeting agents. Linear PEI is more efficient than cationic liposomal formulations *in vivo*. Positively charged PEI polyplexes are endocytosed by cells and PEI is also believed to facilitate endosomal escape. The addition of targeting ligands enhances the activity of PEI presumably by increasing uptake. Recently hydrophobised PEI has been incorporated within DOPE, egg phosphatidylcholine and dipalmitoyl phosphatidylcholine liposomes, producing an efficient gene-transfer agent, although the activity of this soluble amphiphilic polymer was diminished when administered without the liposomal lipids. Unfortunately, PEI, as with some of the cationic lipids, has also been reported to be toxic to cells [14].

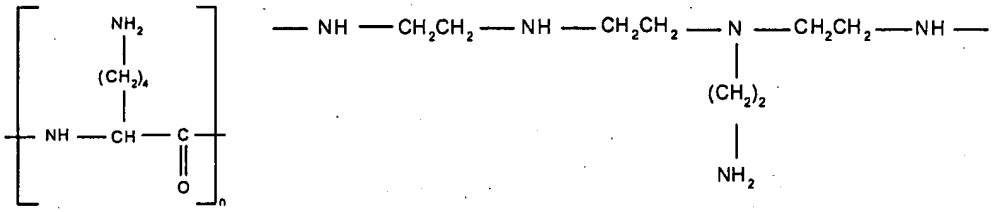

Figure 4: A) Structure of Poly Lysin (PL), B) St ructure of Polyethylenimine (PEI)

Poly (vinylamine) (PVA) was the second type of polycation studied. Molecular weight and dimenstions of DNA complexes formed with PVA were certain degree affected by the molecular weight of the PVA used [4].

Collagen: The first use of polymers to deliver DNA in tissue engineering applications 9 involved the delivery of plasmids encoding for bone morphogenic proteins (BMPs) and fragments of human parathyroid hormone genes. The polymer that used was type I collagen, and the collagen–DNA mixtures were termed gene-activated matrices (GAM)[12].

Liposome/DNA complexe:

There have been several studies on the *in vivo*, systemic use of liposome/DNA complexes. The factors controlling the transfection efficiency of liposome/DNA complexes following IV administration are still poorly understood. The transfection efficiency of liposome/DNA complexes *in vivo* has been shown to be relatively low, especially when compared to viral vectors. One study has suggested that the *in vivo* transfection efficiency of adenoviruses is around 200 times greater than that observed with liposomes. One explanation for the

relatively poor transfection efficiency of liposome/DNA complexes is that they are susceptible to disruption by serum proteins. A variety of proteins are known to bind to liposomes *in vitro* and *in vivo* and may membrane destabilisation. There are now serious efforts being made to develop liposomal vectors that are resistant to serum disruption. Novel cationic lipids are also being developed to try to improve the transfection efficiency of liposome/DNA complexes. Targeting of the liposomes to specific cell types has also been investigated as a means of improving the transfection efficiency.

It was determined that the amount of protein bound to the liposome/DNA complexes is directly proportional to the clearance time of the complexes from the bloodstream. The incorporation of cholesterol into the liposome/DNA formulation, which increases the packing densities of the phospholipid molecules, drastically reduces the amount of bound protein and produces a corresponding improvement in circulation half-life. The incorporation of 30mol % cholesterol increases the half-life of liposome/DNA complexes from seconds to over 5 hours. The amount of serum protein binding to liposome/DNA complexes can also be reduced by increasing the dose of the liposome/DNA complex administered intravenously. This reduction in protein binding produced a corresponding increase in the circulation times, from around 4 minutes to over 80 minutes.

Peptide mediated gene delivery

A related approach is to use naturally occurring or synthetic peptides as gene delivery systems. The interaction of nucleic acids with basic polyelectrolytes is a process that has been known for a long time. In 1946, Kleczkowski showed [10] that the conjugation of proteins with nucleic acids is a general phenomenon that takes place whenever the pH allows them to be of oppisite charges, and also suggested that the interaction of proteins with viral nucleoproteins may be responsible for reducing the infectivity of some viruses.

The importance of serum protein interactions on circulation and transfection activity has been established with cationic liposomes. It is well known that serum has an inhibitory effect on the transfection efficiency of cationic liposome/DNA complexes. Binding of the liposome/DNA complexes to negatively charges serum proteins, leading to a decrease in cell association has been implicated as a mechanism for the inhibitory effect of serum on transfection activity. It has since been demonstrated that this inhibition can, at least partly, be overcome by increasing the charge ratio or dose of the liposome/DNA complex.

The approach of use proteins for DNA delivery is based upon the observation that the functionally active regions of proteins such as enzymes, receptors and antibodies are relatively small, typically consisting of around 10-20 amino acids. Synthesising peptides based upon functional regions of DNA binding proteins or a variety of viral proteins is an approach that has been used to replace the use of whole proteins (such as histone H1) or large, polydispersed polymers (such as polylysine) as gene delivery vectors. A number of peptide sequences have been shown to be able to bind to and condense DNA. One such sequence is the tetra-peptide serine-proline-lysine-lysine located in the C-terminus of the histone H1 protein. Rational design of peptide sequence has also been used to develop completely synthetic DNA binding peptides.

Albumin is another protein that cans interact with DNA complexes by electrostatic or hydrophobic interactions. It has been showed that albumin is not able to decompose the complexes and release free DNA, but it could probably interact with positively charged complexes and form ternary albumin–DNA–polycation complexes. In that case, some parameters of the DNA complexes, such as z-potential or hydrodynamic radius must be changed.

Reference:

[1] David M. Lynn, Daniel G. Anderson, David Putnam, Robert Langer, *Accelerated Discovery of Synthetic Transfection Vectors: Parallel Synthesis and Screening of a Degradable Polymer Library*, J. Am. Chem. Soc., 123(33), (2001) 8155-8156

[2] Luo, D., Saltzman, W. M. Nat. Bio-technol, 18, (2000) 33-37

[3] Lim, Y., Kim, S. et all., J. Am. Chem. Soc. (2001)

[4] Hwang, S. J.; Bellocq, N. C.; Davis, M. E. Bioconjugate Chem. (2001)

[5] A.E.Smith, *viral vectors in gene therapy*, Annu. Rev. Microbila. 49 (1995) 807-838

[6] M.A. Wolfert, Nazarova, L.W. Sey-mour, *Characterization of vectors for gene therapy formed by self-assembly of DNA with synthetic block copolymers*, Hum. Gene Ther. 7 (1996) 2123

[7] D.Oupický, C.Konak, et al., *DNA delivery sytems based on complexes of DNA with synthetic polycations and their copolymers*, Journal of Controlled Release 65 (2000) 149–71

[8] Traian V.Chirila, Ian J.Constable, *The use of synthetic polymers for delivery of therapeutic antisense oligodeoxy-nucleotides*, Biomaterials 23 (2002) 321-42

[9] K. Nishida, H. Sezaki, *Pharmacokinetic analysis of uptake process of lactosaminated albumin in rat liver constant infusion experiments*, Int. J. Pharm. 80 (1992) 101–108.

[10] Kleczkowski, *Combination between different proteins and between proteins and yeast nucleic acid*, Biochem. Journal; 40, (1946) 677-7

[11] Philip R. Dash, Veska Toncheve, L.W. Seymour, *Synthetic polymers for vectorial delivery of DNA: charackteris-ation of polymers-DNA comp-lexes by photon correlation spectroscopy and stability to nuclease degradation and distruption by polyanions in vitro*, J. of Controlled Release 48 (1997) 269-276

[12] Sara-Kaye Madsen and David J. Mooney, *Delivering DNA with polymer matrices: applications in tissue eng-ineering and gene therapy*, PSTT Vol. 3, No. 11 November 2000

[13] Mitsuru Hashida, Yoshinobu Taka-kura, *Cell-specific delivery of genes with glycosylated carriers*, Advanced Drug Delivery Reviews 52 (2001) 187–196

[14] Haeshin Lee, Ji Hoon Jeong, Tae Gwan Park, *A new gene delivery formulation of polyethylenimine / DNA complexes coated with PEG conjugated fusogenic peptide*, Journal of Controlled Release 76 (2001) 183–192

[15] Haensler J, Szoka FC Jr, *Polyamido-amine cascade polymers mediate efficient transfection of cells in culture*. Bioconjug Chem (1993), 4: 372-379

[16] Fred D Ledley, *Non-viral gene therapy*, Current Opinion in Biotechnology (1994) 5: 626-636

[17] M. Nishikawa, M. Yamauchi, K. Morimoto, E. Ishida, Y. Takakura, M. Hashida, *Hepatocyte-targeted in vivo gene expression by intravenous injection of plasmid DNA com- plexed with synthetic multifunctional gene delivery system*, Gene Ther. 7 (2000) 548